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Constitutive expression of the Id-1 promoter in human metastatic breast cancer cells is linked with the loss of NF-1/Rb/HDAC-1 transcription repressor complex

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The helix–loop–helix protein Id-1 is a dominant negative regulator of basic helix–loop–helix transcription factors, and plays a key role in the control of breast epithelial cell growth, invasion and differentiation. Previous investigations in our laboratory have shown that Id-1 mRNA was constitutively expressed in highly aggressive and invasive human breast cancer cells in comparison to non-transformed or non-aggressive cancerous cells, and that this loss of regulation is mediated by a 2.2-kb region of the human Id-1 promoter. Here we show that a 31 bp sequence within this 2.2-kb promoter, located 200 bp upstream of the initiation of transcription, is responsible for the constitutive expression of Id-1 in metastatic human breast cancer cells. Using gel shift experiments, we identified a high molecular weight complex present only in non-aggressive breast cancer cells cultured in serum-free medium and which appear to be necessary for proper Id-1 repression. In contrast, nuclear extracts from highly aggressive and metastatic cell lines do not contain this large molecular weight complex. Using DNA affinity precipitation assays (DAPA), we show that this complex contains SP-1, NF-1, Rb and HDAC-1 proteins. On the basis of these findings, we propose a mechanism for the loss of regulation of Id-1 promoter in invasive and metastatic human breast cancer cells.
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Introduction

When normal breast epithelial cells become transformed, a number of genetic alterations occur which lead to tumorigenesis and metastasis. These alterations affect growth control, maintenance of differentiated epithelial functions and invasiveness. Invasiveness marks the onset of metastasis, which is a hallmark of often fatal malignant progression. Identifying the genes involved in these processes is not only essential for understanding how breast cancers develop and progress, but also for deriving better methods for prognosis and treatment. Our preliminary data indicate the likely involvement of the helix–loop–helix (HLH) protein, Id-1, as a molecular switch not only between growth/invasion and differentiation in mammary epithelial cells, but also during breast cancer progression.

Id proteins ('Inhibitor of differentiation' or 'Inhibitor of DNA binding') are a family of HLH proteins that bind to and inhibit the activity of basic helix–loop–helix (bHLH) transcription factors (Benezra *et al*., 1990; Sun *et al*., 1991). These bHLH transcription factors consist of a large family of ubiquitous as well as tissue specific proteins that can bind to the DNA as homo- or hetero-dimers and activate the transcription of genes important for differentiation (Kadesch, 1993; Littlewood and Evan, 1994; Massari and Murre, 2000). Since Id proteins lack the basic DNA binding domain, the heterodimers that bHLH factors form with the Id proteins are unable to bind to DNA. Consequently, Id proteins act as dominant negative regulators of bHLH transcription factors by sequestering the ubiquitous bHLH proteins and preventing their association with tissue specific bHLH transcription factors (Jen *et al*., 1992; Neuhold and Wold, 1993). Id family members have been shown to be preferentially expressed in proliferating cells and down-regulated in differentiating cells and ectopic expression of Id proteins has been shown to inhibit differentiation of a wide variety of cell types (reviewed in Norton *et al*., 1998; Norton, 2000).

We have previously shown that constitutive expression of Id-1 inhibits the functional differentiation of the murine mammary epithelial cell line SCp2 (Desprez *et al*., 1995) and stimulates the proliferation and invasiveness of these cells (Desprez *et al*., 1998; Singh *et al*., 2001). In addition, we observed a strong correlation between the levels of Id-1 expression and the levels of aggressiveness and invasiveness of several human breast cancer cell lines (Desprez *et al*., 1998; Lin *et al*., 2000). Poorly differentiated and highly metastatic breast cancer cells such as MDA-MB-231 and MDA-MB-436 express Id-1 mRNA constitutively and have

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lost serum dependent regulation of the Id-1 gene as compared to non-transformed mammary epithelial cells (Desprez *et al*., 1995) or well-differentiated and non-aggressive breast cancer cells such as T47D and MCF-7 (Desprez *et al*., 1998). Moreover, T47D cells with ectopic Id-1 expression become invasive and show a higher proliferation index than control cells (Lin *et al*., 2000). Analysis of Id-1 expression in tumor biopsies also reveals that its expression is up-regulated in infiltrating ductal carcinomas in comparison to ductal carcinoma *in situ* (Lin *et al*., 2000). Higher levels of Id-1 gene expression have also been detected in different types of tumor cells when compared to normal cells of the same tissue origin (Maruyama *et al*., 1999; Kebebew *et al*., 2000; Langlands *et al*., 2000; Takai *et al*., 2001; Ouyang *et al*., 2001; Hu *et al*., 2001; Polsky *et al*., 2001; Schindl *et al*., 2001).

Preliminary analysis of the human Id-1 promoter in T47D and MDA-MB-231 cell lines confirmed that highly aggressive MDA-MB-231 cells have lost serum dependent regulation as compared to non-aggressive T47D cells (Lin *et al*., 2000). In the present study, we attempted to elucidate some of the mechanisms by which metastatic breast cancer cells lose proper Id-1 regulation. We show that two DNA regions are important for regulation of the Id-1 promoter. One region is located 1.2 kb upstream of the transcription start and contains Egr-1 as well as YY-1 and CREB/ATF consensus sequences. This region is responsible for the serum responsiveness of the Id-1 promoter, in close agreement with the previous findings on the mouse Id-1 promoter (Tournay and Benezra, 1996). Additionally, there is a 31 bp sequence located 200 bp upstream of the Id-1 transcription start. This sequence is responsible for constitutive expression of Id-1 in highly aggressive breast cancer cells. Based on results using electrophoretic mobility shift assays and DNA affinity precipitation assays, we propose a mechanism for differential regulation of Id-1 promoter in aggressive and non-aggressive human breast cancer cells.

Results

Serum responsive element in the human Id-1 promoter

We have previously shown in our laboratory that highly aggressive human breast cancer cells have lost serum dependent regulation of Id-1 gene, resulting in constitutive expression of Id-1 mRNA and thus increased proliferative and invasive capacities (Desprez *et al*., 1998; Lin *et al*., 2000; Singh *et al*., 2001). In contrast, normal and non-aggressive cancerous breast cells retain serum dependent regulation of Id-1 gene expression. In order to characterize the sequence elements responsible for serum dependent expression of the Id-1 gene, we constructed a series of 5′ deletions of the 2.2 kb Id-1 promoter and fused it to the luciferase reporter in PGL-3 basic vector (Id-1-sbsluc promoter represented in Figure 1a). Constructs were transiently transfected in non-aggressive T47D cells. Luciferase activity was high in proliferating cells, but decreased about sixfold after serum-deprivation. The sequences located upstream of −1.2 kb could be removed without much loss of serum responsiveness (compare Id-1-sbsluc, 5′del-1 to 5′del-3 in Figure 1b). However, the removal of an additional 270 bp fragment between −1200 to −932 resulted in a dramatic decrease of serum responsiveness (compare 5′del-3 (70% of full promoter expression) and 5′del-4 (less than 20% of full promoter expression) in Figure 1b).

Identification of sequences responsible for constitutive expression of Id-1 promoter in metastatic breast cancer cells

When cultured either in the presence or absence of serum, aggressive MDA-MB-436 breast cancer cells displayed the same high levels of promoter Id-1-sbsluc activity (Figure 1c). There was no effect of removing a 1 kb sequence from the 5′ end of the promoter on luciferase activity (5′del-1 to 5′del-3). Further deletions (5′del-4 to 5′del-6) had positive effects on the promoter activity. A peak of activity was obtained with the 5′del-6 construct which exhibited nearly twice the activity of the full promoter. However, further deletion creating the minimal promoter construct 5′del-7, dramatically down-regulated the luciferase expression. This indicated that the sequence located between −272 and −145 bp was responsible for constitutive expression of Id-1 gene in metastatic MDA-MB-436 cells.

In order to determine whether or not this differential activity of the Id-1 promoter construct was restricted to T47D and MDA-MB-436 breast cancer cells, we examined luciferase activity of the full promoter, as well as the 5′del-6 promoter, in one other aggressive (MDA-MB-231) as well as two other non-aggressive (MCF-7 and ZR-75) breast cancer cell lines. The non-aggressive cell lines MCF-7 and ZR-75 behaved like T47D (Figure 2). Luciferase expression of the full Id1sbs-luc promoter was dramatically decreased when cells were serum-deprived, and the low level of expression was maintained with the 5′del-6 promoter construct. Highly invasive and metastatic MDA-MB-231 cells displayed the same pattern of expression as MDA-MB-436, although the increase of luciferase activity with 5′del-6 was not as pronounced as in MDA-MB-436 (Figure 2). In both aggressive cell lines, high levels of expression were lost using the shortest 5′del-7 construct. These results confirm that the sequence elements responsible for constitutive Id-1 expression in metastatic breast cancer cell lines are located between −272 to −145 bp upstream of the transcription start site.

In order to confirm that the serum response element was located between −1200 and −932 bp, and that the sequence element responsible for constitutive Id-1 expression in metastatic breast cancer cells was located between −272 and −145 bp, we designed three additional deletion constructs derived from the full promoter (Figure 3a). The first construct had a deletion in the region between −1361 and −1200 bp

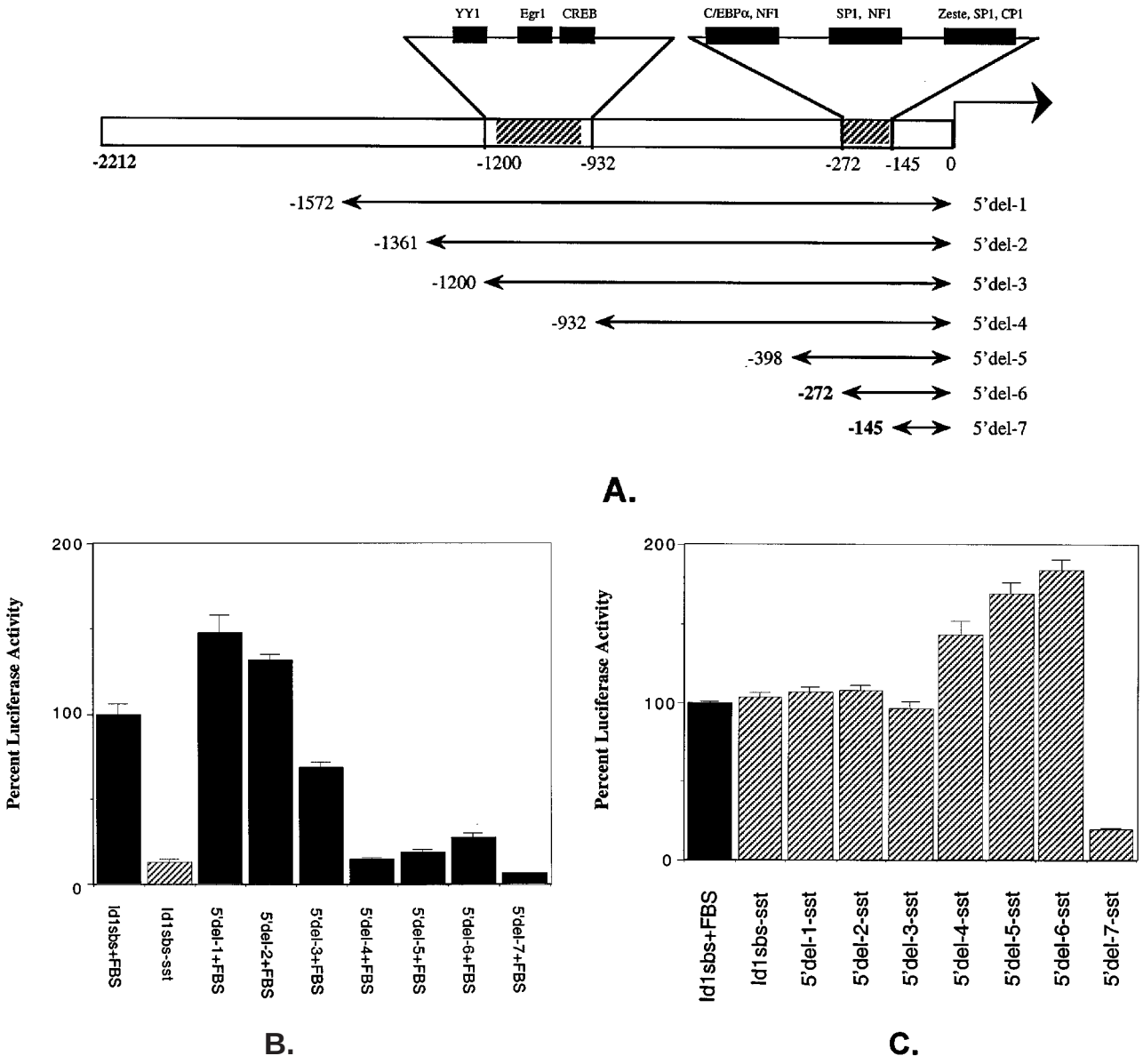

Figure 1 (**a**) Schematic representation of 5′ deletions of the human Id1sbs promoter. Deletion of the region (shaded) between −1200 and −932 bp resulted in loss of serum responsiveness in non-aggressive human breast cancer cells. Deletion of the region (shaded) between −272 and −145 bp resulted in loss of the promoter activity in aggressive and invasive breast cancer cells. (**b**) 5′ deletion analysis of the human Id-1 promoter region in T47D breast cancer cells. Cells were transfected with the 2.2 kb Id1sbs promoter (fused to luciferase) as well as the indicated 5′ deletion mutants as shown in (**a**) along with a CMV-β-gal construct as an internal control. Cells were either maintained in high serum (FBS) or deprived of serum (sst) for 48 h prior to harvesting. All values are normalized for the amount of β-gal activity present in the cell extracts. The results are representative of three independent transfection experiments. (**c**) 5′ deletion analysis of the human Id-1 promoter region in MDA-MB-436 breast cancer cells. Cells were transfected with the 2.2 kb Id1sbs promoter (fused to luciferase) as well as the indicated 5′ deletion mutants as shown in (**a**), along with a CMV-β-gal construct as an internal control. Cells were either maintained in high serum (FBS) or deprived of serum (sst) for 48 h prior to harvesting. All values are normalized for the amount of β-gal activity present in the cell extracts. The results are normalized for the amount of β-gal activity present in the cell extracts. The results are representative of three independent transfection experiments

(Id1sbs-Δ1) and was used as a control. The second construct was deleted in the serum response region between −1200 and −932 (Id1sbs-Δ2) whereas the third one was deleted in the region involved in the constitutive Id-1 expression between −272 and −145 (Id1sbs-Δ3). These different constructs were used in promoter assays either in the presence of serum (for non-aggressive T47D and MCF-7 cells) or in the absence of serum (for the aggressive and invasive MDA-MB-231 and MDA-MB-436 cells). The data presented in Figure 3b confirmed that the serum responsive element was located between −1200 to −932 bp on Id-1 promoter. In T47D and MCF-7 cells, there was a 10–15-fold decrease in luciferase activity

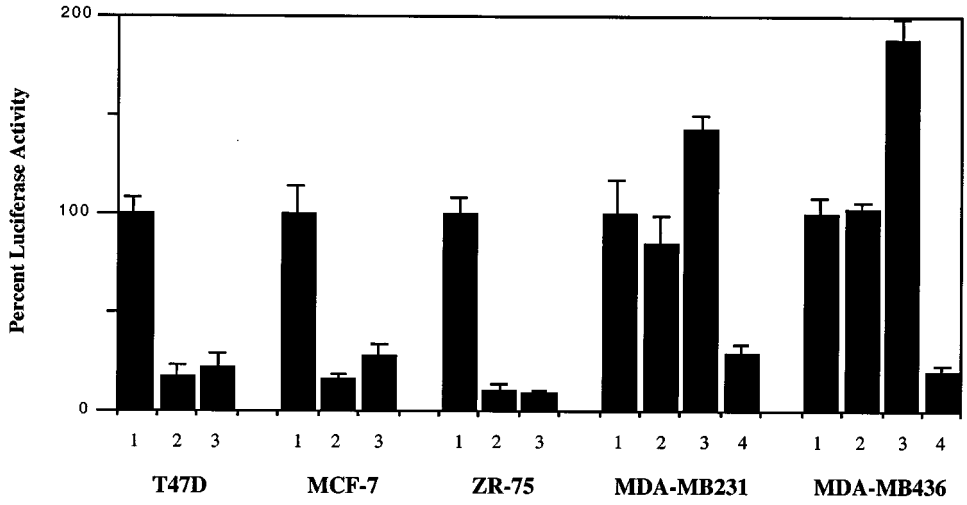

Figure 2 Promoter analysis of various non-aggressive (T47D, MCF-7 and ZR-75) and highly aggressive and invasive (MDA-MB-231 and MDA-MB-436) breast cancer cell lines using full promoter (Id1sbs) or 272 bp 5′ del-6 promoter. Lane 1: 2212 bp Id1sbs promoter construct (high serum). Lane 2: 2212 bp Id1sbs promoter construct (serum deprived). Lane 3: 5′ del-6/272 bp promoter construct (presence of serum for T47D, MCF-7 and ZR-75; absence of serum for MDA-MB-231 and MDA-MB-436). Lane 4: 5′ del-7/145 bp promoter construct (serum deprived). The data are represented as percentage of activity of the full 2212 bp promoter in cells treated with serum

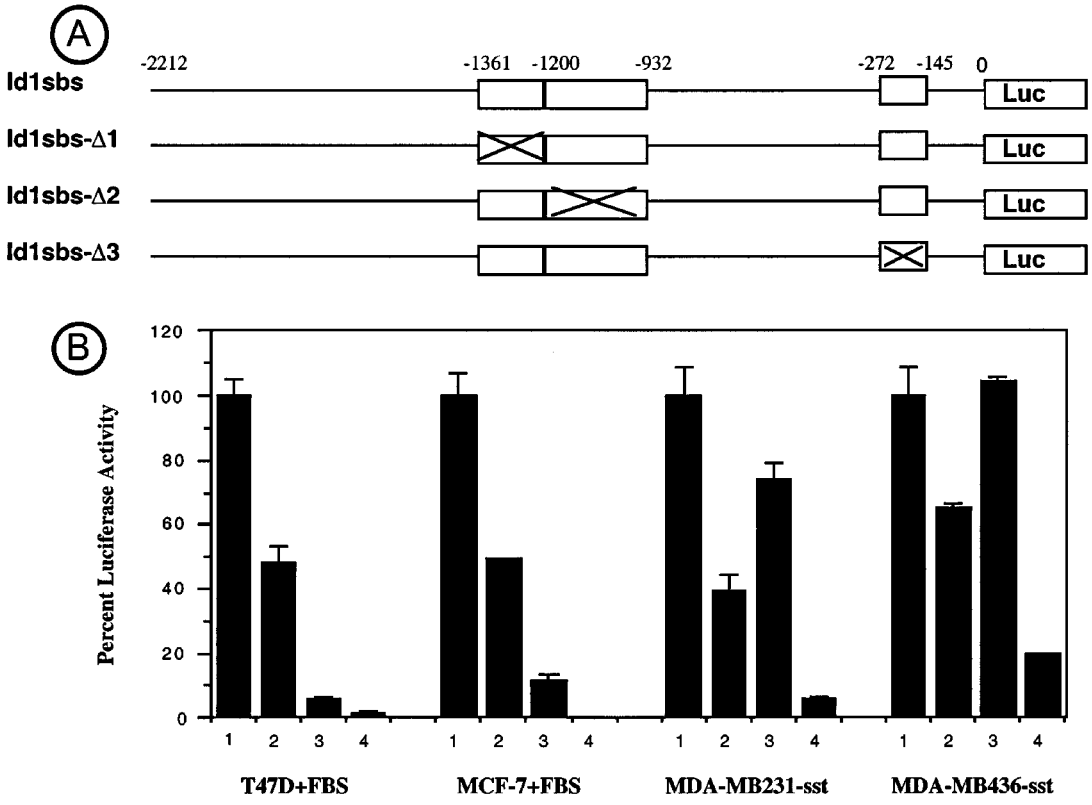

Figure 3 (**a**) Representation of internal deletions made in the Id1sbs promoter construct. Deletion in a particular element is indicated by a X in that box. (**b**) Effect of internal deletions in Id1sbs promoter on the luciferase activity in non-aggressive (T47D, MCF-7) and highly aggressive and invasive (MDA-MB-231 and MDA-MB-436) breast cancer cell lines. T47D and MCF-7 cells were cultured in the presence of serum (FBS), whereas MDA-MB-231 and MDA-MB-436 cells were cultured in the absence of serum (sst). Lane 1: Id1sbs promoter construct; Lane 2: Id1sbs-Δ1 promoter construct; Lane 3: Id1sbs-Δ2 promoter construct; Lane 4: Id1sbs-Δ3 promoter construct. The data are presented as percentage of activity of the full 2212 bp promoter

for Id1sbs-Δ2 in comparison to the full Id1sbs promoter. Id1sbs-Δ1 showed a decrease in luciferase activity to only about half of that of cells with the full promoter. This result was in agreement with the one described in Figure 1b using the 5′del-3 construct (30 to 40% decrease in activity compared to the full promoter). This may indicate the presence of some minor regulatory elements upstream of −1200 bp. However, using the Id1sbs-Δ3 construct, we found a dramatic decrease of the luciferase activity in all four cell lines tested. This result indicated that the −272 to −145 region (127 bp segment) was not only crucial for constitutive Id-1 expression in metastatic cells, but also for serum responsiveness in non-aggressive breast cancer cells.

Localization and characterization of the novel sequence element responsible for constitutive Id-1 promoter activity

In the next set of experiments, we attempted to precisely locate the sequence within the 127 bp segment that was responsible for the constitutive expression of the Id-1 promoter in metastatic breast cancer cells. Using the database 'TRANSFAC' to identify the different transcription factor binding sites, we localized three different clusters. The first one, between −262 to −252 (Box-1), contained binding sites for C/EBPα and NF-1; the second one, between −203 to −179 (Box-2), contained binding sites for SP-1 and NF-1; and the third one, between −165 to −145 (Box-3), contained binding sites for Zeste, SP-1 and CP-1 (Figures 1a and 4). Using the 5′del-6 construct (272 bp), we prepared three deletion constructs (5′del-6-δ1 to δ3), each missing one of the three clusters (Figure 4a). These three vectors, along with 5′del-6, were used in promoter assays in serum-treated T47D, and in serum-deprived MDA-MB-231 and MDA-MB-436 cells. In all three cell lines tested, promoter activities decreased significantly when we used the 5′del-6-δ2 vector (Figure 4b). The region deleted in this vector corresponded to the two binding sites for SP-1 and NF-1.

To confirm the role of Box-2 in the regulation of Id-1 gene expression, we fused it to the 5′del-7 minimal promoter (5′del-7 + Box-2) and compared the activity of this new construct to the activity of the 5′del-6 and 5′del-7 promoter constructs. We used two cell lines for this experiment. In the first one, T47D, the expression of the 5′del-6 construct was shown to be about 25–30% of that of the full promoter (in the presence of serum). In the second cell line, MDA-MB-436, the expression of the 5′del-6 construct was shown to be about 180% of that of the full promoter (in the absence of serum). In both cell lines, luciferase activity was dramatically reduced using the 5′del-7 construct. However, in both cell lines tested, addition of Box-2 to the minimal promoter dramatically increased its activity (Figure 4c). The luciferase activity was about 50–60% of that of the 5′del-6 construct, indicating that Box-2 is playing a major role in the activation of the Id-1 promoter. However, there may be other minor sequences in Box-1 and Box-3 that may also participate in the regulation of the Id-1 promoter.

Electrophoretic mobility shift assay of the sequence element responsible for constitutive Id-1 expression

In order to further characterize the binding elements, we performed electrophoretic mobility shift assays (EMSA) using nuclear extracts from the non-aggressive as well as aggressive breast cancer cell lines. We first used a 31 bp double stranded oligonucleotide spanning the box-2 region between −200 and −169 bp and containing SP-1 and NF-1 sites (Figure 5a). Using nuclear extracts from T47D, a high molecular weight complex was only detectable in cells cultured in serum-free medium and not in 10% serum (Figure 5c). Nuclear extracts from MCF-7 cells cultured in the absence of serum also displayed the same high molecular weight complex which was absent from nuclear extracts isolated from the two metastatic MDA-MB-231 and MDA-MB-436 cell lines (Figure 5d). We then used the mutated oligonucleotides described in Figure 5b mixed with nuclear extracts from serum-deprived T47D cells. As shown in Figure 5e, no high molecular weight complex was detectable using oligonucleotides with mutations in SP-1 and NF-1 sites. On the other hand, mutations in the 5′ or 3′ end of the 31 bp oligonucleotide had no or little effect on the formation of this complex. Therefore, the sequences flanking the SP-1 and NF-1 sites are probably not involved in the formation of the HMW complex.

In addition, we used mutant 31-mer oligonucleotides as competitors for the EMSA. One of the oligos was mutated in the SP-1 site and the other one in the NF-1 site. In the competition experiments using 1000-fold excess of unlabeled oligos, only the wild type 31-mer could inhibit the formation of the HMW complex (Figure 5f). Using either mutated SP-1 (mSP-1) and mutated NF-1 (mNF-1) oligonucleotides, we could not prevent the formation of the HMW complex. In the case of mNF-1, the amount of HMW complex formed was almost identical to that without any competition. These results suggest that the NF-1 site is the critical one for the formation of the HMW complex, although the SP-1 site also appears to contribute significantly to HMW complex formation.

Identification of the proteins involved in the high molecular weight complex

We first attempted to detect the presence of NF-1 in the complex using supershift analysis. No higher molecular weight complex was detectable (data not shown). The absence of a supershifted band has been described in other systems. For example, preincubation of nuclear extracts from exponentially-growing PC12 cells with an antiserum directed against p300 (a co-activator also involved in a multi-protein complex) did not cause any complex to supershift (Billon *et al*., 1999). As reported previously, this phenomenon may be due to epitope masking (Avantaggiati *et al*., 1997). Therefore, in order

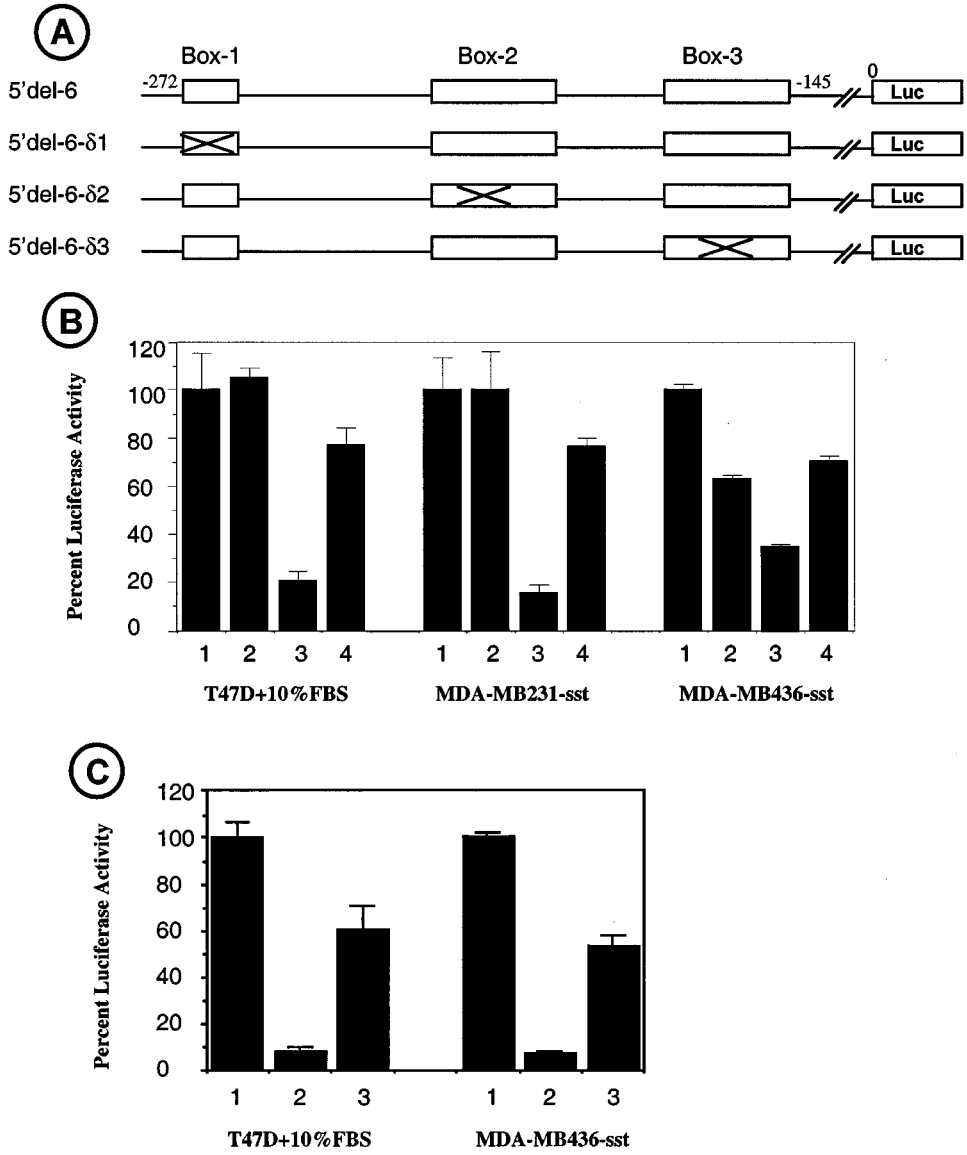

Figure 4 Schematic diagram of internal deletions made in 5′ del-6 promoter construct used in transient transfection assays. Deletion in a particular element is indicated by an X in that box. Box-1 contains C/EBPα and NF-1 response elements; Box-2 contains SP-1 and NF-1 sites; Box-3 contains Zeste, SP-1 and CP-1 sites. (**b**) Luciferase activity in various cell lines transiently transfected with 5′ del-6 (lane 1) or 5′ del-6-δ1 (lane 2), 5′ del-6-δ2 (lane 3) and 5′ del-6-δ3 (lane 4). The cells were harvested 48 h after transfection and cultured either in presence of serum (T47D) or in the serum starved condition (MDA-MB-231 and MDA-MB-436). The data are presented as percentage of activity of the 5′ del-6 promoter. (**c**) Luciferase activity in cells transiently transfected with 5′ del-6 (lane 1), 5′ del-7 (lane 2) or 5′ del-7 + Box-2 (lane 3). The cells were cultured either in the presence of serum (T47D) or in serum-starved conditions (MDA-MB-436) and were harvested 48 h after transfection. The data are presented as percentage of activity of the 5′ del-6 promoter

to identify the components involved in the formation of the high molecular weight complex, we chose to perform DNA affinity precipitation assays using the biotinylated 31-wt oligonucleotide. Nuclear extracts from T47D as well as MDA-MB-436 cells cultured in presence or absence of serum were used in these assays and incubated with the oligonucleotide. Western blot analysis showed that all four samples displayed the same amount of a 55 kDa SP-1 protein, irrespective of the presence or absence of serum (Figure 6). However, a 46 kDa band corresponding to NF-1 was only detectable in serum-deprived T47D. Samples from MDA-MB-436 cells did not show any binding of NF-1 under any culture condition. Further analysis of the DAPA Western blots using anti-histone deacetylase 1 (HDAC-1) as well as anti-Rb antibodies, suggested that both HDAC-1 and Rb were part of the large molecular weight complex binding to the 31 bp sequence. Protein–protein interactions were confirmed by the observation that HDAC-1 and Rb could be precipitated from the cell extracts of serum-deprived T47D by co-immunoprecipitation with anti-NF-1 as well as anti-SP-

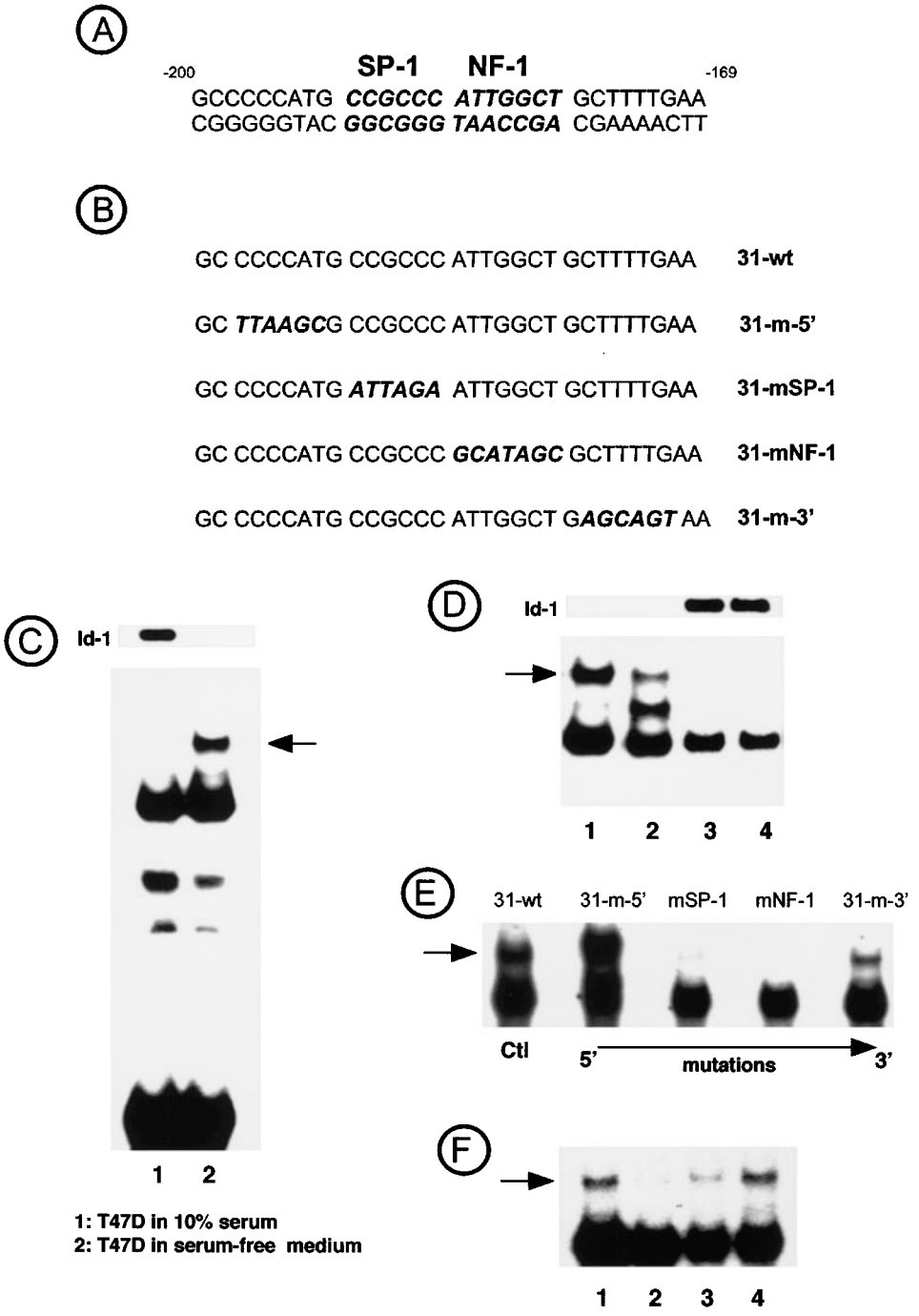

Figure 5 Electrophoretic mobility shift assay (EMSA) using the 31 bp double stranded wild type or mutated oligonucleotides. Nuclear extracts from various breast cancer cell lines cultured either in presence or absence of serum were incubated with ^{32}P-labeled oligos spanning the region −200 to −169. The position of the specific protein complex that binds to these oligos after serum starvation is indicated by the arrow. (**a**) Sequence of the GC rich Box-2 in the human Id-1 promoter showing one consensus sequence element for SP-1 and one for NF-1 transcription factors. (**b**) Sequences of the oligonucleotides used for electrophoretic mobility shift assays. The 31-wt oligo contains the SP-1 site and the NF-1 site. Other oligos have mutations (shown in bold) either in one of the two sites (mSP-1 or mNF-1) or in their 5′ or 3′ region. (**c**) EMSA of nuclear extracts prepared from T47D cultured in 10% serum (lane 1) or serum-starved (lane 2) using the wt-31mer oligo. (**d**) EMSA of nuclear extracts prepared from four different breast cancer cell lines in serum-starved conditions using the wt-31mer oligo. Lane 1: T47D; lane 2: MCF-7; lane 3: MDA-MB-231; lane 4: MDA-MB-436. (**e**) EMSA of nuclear extracts prepared from serum-starved T47D using the wt-31 mer oligo as well as various mutated oligos (as described in **b**). (**f**) EMSA of nuclear extracts prepared from serum-starved T47D cells using the wt-31mer oligo in the absence (lane 1) or the presence of 1000-fold molar excess of either wt (lane 2), mSP-1 (lane 3) or mNF-1 (lane 4) unlabeled oligonucleotides

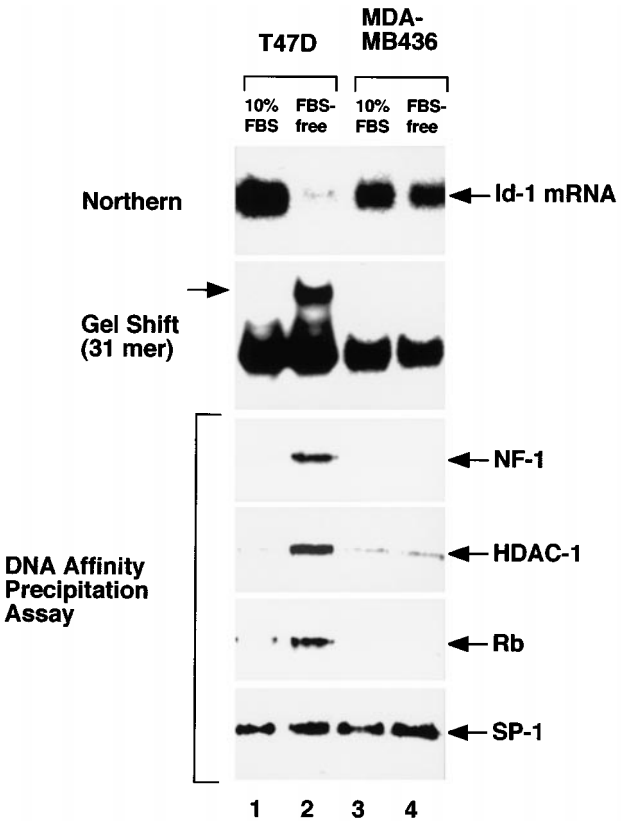

Figure 6 DNA affinity precipitation assays (DAPA) using the 31 bp oligonucleotide. DAPA was performed by incubating biotinylated 31-wt oligonucleotides (described in the EMSA studies) with nuclear extracts from T47D and MDA-MB-436 cells cultured either in presence of 10% serum or in serum-starved condition. Lanes: (1) T47D + FBS; (2) T47D-sst; (3) MDA-MB-436 + FBS; (4) MDA-MB-436-sst. The affinity precipitated complexes were denatured in sample buffer and SDS–PAGE was performed, the gel transferred to nitrocellulose membrane, followed by Western blotting using specific antibodies against NF-1, HDAC-1, Rb and SP-1. The first and second panels from the top show Id-1 mRNA expression and gel shift assay, respectively, from the cells cultured under similar conditions as DAPA

1 antibodies (Figure 7a). Finally, we determined by Western blot analysis of whole nuclear extracts that non-aggressive T47D and MCF-7 cell lines expressed NF-1, HDAC-1 and Rb proteins. On the other hand, NF-1 was not expressed in MDA-MB-231 cells, and NF-1 as well as Rb were absent in extracts from MDA-MB-436 (Figure 7b).

Discussion

Previous investigations in our laboratory have shown that constitutive expression of Id-1 in non-transformed mouse mammary epithelial cells and in non-aggressive breast cancer cells results in an invasive phenotype in addition to a higher proliferation rate (Desprez *et al*., 1998; Lin *et al*., 2000; Singh *et al*., 2001). Moreover, highly invasive and metastatic human breast cancer cells, in contrast to non-aggressive cells, constitutively express Id-1 losing serum-dependent regulation of Id-1 promoter. We hypothesize that this deregulated expression of Id-1 is one of the major causes for the development of a metastatic phenotype in breast cancer cells. This hypothesis is also supported by our findings that infiltrating breast carcinomas express much higher levels of Id-1 protein than do ductal carcinomas *in situ* (Lin *et al*., 2000). Moreover, another group has reported that Id-1 and Id-3 knockout mice have impaired invasion and angiogenesis capabilities (Lyden *et al*., 1999). Understanding these observations requires study of the mechanisms by which metastatic cancer cells lose proper Id-1 regulation.

Using the mouse Id-1 promoter, earlier reports identified a sequence containing Egr-1 and YY-1 sites responsible for serum dependent up-regulation of the promoter (Tournay and Benezra, 1996). Our data using deletion analysis of the human Id-1 promoter in T47D cells confirmed the presence of a serum responsive element located in the region between −1200 to −932 bp, a region containing binding sites for Egr-1 and YY-1 as well as CREB/ATF transcription factors. However, in addition to this serum-responsive element, we identified a 127 bp region located at the 3′ end of Id-1 promoter that was necessary for serum-dependent regulation in non-aggressive cells, and, more importantly, for constitutive Id-1 expression in metastatic breast cancer cells.

Sequence analysis of this 3′ region indicated that it contained three clusters of transcription factor binding sites. Further deletion analysis indicated that the second cluster, a 31 bp sequence containing SP-1 and NF-1 sites, appeared to mediate the constitutive expression of Id-1. Both sites were necessary for the formation of a high molecular weight (HMW) complex in the gel shift experiments. This HMW complex was present only in nuclear extracts from non-aggressive breast cancer cells serum-deprived, and therefore in cells which did not express Id-1 gene. We therefore hypothesize that this complex, absent in metastatic cells, is responsible for the normal repression of Id-1 gene transcription in the absence of serum, growth factors and/or steroid hormones.

One of the two factors, SP-1, has been shown to bind to the co-repressor molecule histone deacetylase 1 in resting cells, an interaction decreased upon serum stimulation (Doetzlhofer *et al*., 1999). Histone acetylases (HAs) and deacetylases (HDACs) are chromatin remodeling agents (Wade *et al*., 1998; Zhang *et al*., 1998; Burke and Baniahmad, 2000) which modulate gene transcription in coordination with other sequence specific transcription factors. Acetylation of nucleosomal histones at N-terminal lysine residues by HAs can lead to decondensation of the chromatin due to decreased affinity between DNA and histone molecules. This may result in higher accessibility of these DNA regions to transcriptosomes, leading to increased transcription of the target genes (Grunstein, 1997; Kuo and Allis, 1998; Wade and Wolffe, 1997). Conversely, HDACs act as repressors of transcription by condensing the chromatin structure, thereby making

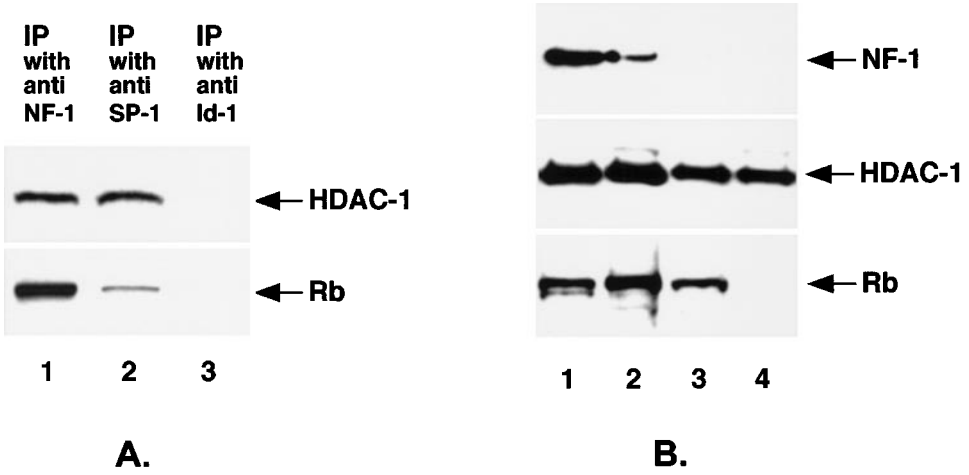

Figure 7 (**a**) Interaction of NF-1 and SP-1 with HDAC-1 and Rb proteins. Total cell extracts from serum-starved T47D cells were immunoprecipitated with either anti-NF-1 (lane 1), anti-SP-1 (lane 2) or anti-Id-1 (lane 3 as negative control) antibodies. Immunoprecipitated complexes were resolved on SDS–PAGE, transferred to nitrocellulose membrane and subjected to Western blotting with anti-HDAC-1 or anti-Rb antibodies. (**b**) Whole nuclear extracts from serum-starved T47D (lane 1), MCF-7 (lane 2), MDA-MB-231 (lane 3), and MDA-MB-436 (lane 4) cells were subjected to Western blotting and incubated with antibodies against NF-1, HDAC-1 and Rb

it inaccessible to the transcriptional machinery (Pazin and Kadonaga, 1997; Wolffe, 1996). Our data from the DNA affinity precipitation experiments indicate that HDAC-1 binding to the SP-1/NF-1 complex increases only in serum-deprived T47D cells, but not in serum-deprived metastatic cells.

Interestingly, we found that Rb protein was also part of this HMW complex. Rb protein has been reported to repress E2F-regulated promoters by recruiting HDACs (Magnaghi-Jaulin *et al*., 1998; Brehm *et al*., 1998). Thus, Id-1 may represent another gene for which a HDAC1-Rb interaction is necessary for proper transcriptional control. Failure to repress transcription of Id-1 by HDAC-1 due to loss of Rb expression might therefore be one of the mechanisms leading to tumor cell progression (Wade, 2001). It is worth noting that, in one of the two metastatic cell lines, Rb protein expression was undetectable by Western blotting. We do not know the mechanisms responsible for the loss of expression in these cells. Nevertheless, this is in agreement with the aberrant Rb gene expression reported in a subset of aggressive breast cancer tissues (Tamura *et al*., 1994; Geradts *et al*., 1994). This loss of Rb expression may contribute to the loss of Id-1 gene repression and therefore to breast cancer progression.

The other factor present in this complex, NF-1, showed a high binding affinity for the 31 bp sequence using nuclear extracts of T47D cells cultured in serum-free medium. On the other hand, we could not detect any NF-1 binding to the 31 bp sequence using nuclear extracts of MDA-MB-436. Since our co-immunoprecipitation experiments revealed that NF-1 could also interact with HDAC-1, we propose that NF-1 is able to recruit HDAC-1 (as well as Rb) to repress Id-1 gene transcription. This is in agreement with the previously described role of NF-1 not only as an activator of transcription, but also as a repressor of transcription, depending on the site it binds to as well as its interaction with various co-factors (Gronostajski, 2000). Since SP-1 is expressed in non-invasive as well as in metastatic cells, we hypothesize that it represents the factor that activates Id-1 gene transcription. However, since NF-1 is absent in aggressive breast cancer cells, NF-1 probably plays the key role in Id-1 repression. Not only we could not detect any NF-1 protein bound to the 31 bp oligonucleotide using the DAPA technique, but we could not detect any NF-1 protein using whole nuclear extracts from the metastatic MDA-MB-231 and MDA-MB-436 cells.

We suggest that NF-1 protein may be lost in a high proportion of metastatic breast cancer cells and that, consequently, these aggressive cells lose the ability to down-regulate Id-1 gene and maintain a high rate of proliferation, invasion and migration. In agreement with this hypothesis, a depletion or low level of NF-1 was found in breast tumor samples (Nayak and Das, 1999).

Moreover, down-regulation of the metastasis tumor suppressor gene nm23 has been reported in human tumor cells with high metastatic phenotype. Using the nm23 promoter, it has been shown that the presence of NF-1 was essential for promoter activity (Chen *et al*., 1994). Therefore, through its ability to down-regulate Id-1 and to maintain high nm23 expression, NF-1 may represent a key factor for the prevention of human breast cancer invasion and metastasis.

Materials and methods

Cell culture

The human breast cancer cell lines T47D, MCF-7, ZR-75, MDA-MB-231 and MDA-MB-436 were purchased from American Type Culture Collection and were maintained in

RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 5 μg/ml insulin. For serum-deprived experiments, cells were maintained in RPMI-1640 medium containing 0.1% FBS and 5 μg/ml insulin.

Promoter-reporter vectors construction

A *Sac*I – *Bsp*HI fragment of 2.2 kb corresponding to the 5′ upstream region of human Id-1 gene and driving a luciferase gene in a PGL-3 vector (Promega) has already been described (Id-1-sbsluc) (Nehlin *et al*., 1997; Lin *et al*., 2000). The 5′ deletion constructs (shown in Figure 1a) were made from this vector by digestion with *Sac*I on the 5′ end and with *Bst*XI (5′del-1), *Eco*RI (5′del-2), *Bst*EII (5′del-3), *Pvu*II (5′del-4), *Bsu*36I (5′del-5) or *Sma*I (5′del-6) at the 3′ end. The digested vector-promoter fragments were isolated from the gel, blunted with T4 polymerase (New England Biolabs) and ligated using T4 DNA Ligase (Promega). The ligated vectors were transfected into DH5α competent cells (Gibco – BRL).

For preparing 5′del-7, PCR was performed on Id-1-sbsluc promoter vector using primer pSac-I 5′-**ATGAGCTC**-CCGTGGCGTGTTTATAAAAGAC-3′ (encompassing region between -145 to -123 bp of the Id-1 promoter and having a *Sac*I site (bold) at the 5′ end) and pNar-I 5′-GATAGAATGGCGCCGGGCCTT-3′ (complementary to the vector region immediately after the MCS site). The resulting product along with Id-1-sbsluc was digested with *Sac*I and *Nar*I. The digested vector and PCR product were ligated and transfected into DH5α and the positive clones were selected for maxi-prep.

Id1sbs-Δ1 and Id1sbs-Δ2 were prepared by internal deletions of either *Eco*RI – *Bst*EII fragment (for Id1sbs-Δ1) or *Bst*EII – *Pvu*II fragment (for Id1sbs-Δ2) from the full Id-1-sbsluc. For Id1sbs-Δ3, PCR was done to amplify Id-1 promoter fragment between -2212 to -266 bp by primers 5′-ATGAGCTCCTTTCTTTAGAGTTG-3′ and 5′-AC-CCGGGTTTCAGAACAGCAA-3′ using Pfu-Turbo DNA polymerase (Stratagene). The amplified fragment was digested with *Sac*I and *Sma*I and ligated to *Sac*I digested 5′Del-7 vector construct.

For the construction of 5′del-6 vectors with deletions δ1 to δ3, inverse PCR was performed (Kaluz *et al*., 1999) to delete the δ1 – δ3 clusters. Briefly, 5′del-6 vector was used as a template for PCR using primer pairs 5′-CTGCGAGCAGG-CACTAGACGA-3′ and 5′-CCTATCGATAGAGAAATG-TTC-3′ for 5′del-6-δ1; 5′-GCTTTTGAACGTTCTGAG-CCC-3′ and 5′-GACCCGCGCTCCTAGGTCCAG-3′ for 5′del-6-δ2; and 5′-CCGTGGCGTGTTTATAAAAGA-3′ and 5′-AACGTTCAAAAGCAGCCAATG-3′ for 5′del-6-δ3. PCR products were ligated and used to transform DH5α. Positive clones were sequenced to verify the intended deletion.

Id-1 promoter reporter assays

The different human breast cancer cells were plated in six well dishes at a density of 3×10^5 cells per well in RPMI-1640 medium supplemented with 10% FBS and 5 μg/ml insulin. After 24 h, cells were cotransfected with 6 μg of luciferase reporter plasmids and 2 μg of pCMVβ (Clontech) using superfect reagent (Qiagen). pCMVβ contained bacterial β-galactosidase driven by the constitutive CMV promoter, and served to control for variation in transfection efficiency. Three hours after transfection, the cells were rinsed twice with PBS and were cultured either in high serum (10% FBS and 5 μg/ml insulin) or in low serum (0.1% FBS and 5 μg/ml insulin) for 48 h before harvest. Cells were scraped into 1 ml of PBS and collected by centrifugation (13 000 r.p.m. for 10 min). Cell pellets were re-suspended in 80 μl of reporter lysis buffer (Promega) for 10 min at room temperature. After centrifugation, supernatants were harvested. Luciferase and β-gal assays were performed using Luciferase Assay System (Promega), β-Gal Assay Kit (Clontech) and a 2010 luminometer (Pharmingen). Luciferase activities were normalized for β-gal activity.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from the various breast cancer cell lines were obtained by modification of the method of Dignam *et al*. (1983). Briefly, cells were scraped and pelleted into PBS, washed in buffer A (10 mM HEPES, 1.5 mM $MgCl_2$, 5 mM KCl, 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM dithiothreitol (DTT) and a cocktail of protease inhibitors), resuspended in buffer A plus 0.1% NP-40, disrupted by 20 strokes in a dounce homogenizer and centrifuged. Nuclear pellets were re-suspended in buffer B (20 mM HEPES, 25% glycerol, 450 mM KCl, 1 mM EDTA and protease inhibitor cocktail), gently agitated at 4°C for 45 min, and spun at 13 000 r.p.m. for 30 min at 4°C. Supernatant were collected, assayed for protein content (Biorad microassay kit) and aliquots were frozen at -70°C until further use.

For gel shift experiments, 5 pmol of double stranded oligonucleotides (individual sequences shown in Figure 5) were end labeled by γ-^{32}P using T4 polynucleotide kinase (New England Biolabs). Aliquots of 3 – 5 μg of nuclear extract were incubated with 10 000 c.p.m. of double stranded oligonucleotides in binding buffer (20 mM HEPES, 80 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT; pH 7.9) containing 1 μg poly dI-dC or poly dA-dT. The mixture was incubated for 20 min at room temperature (RT) and then loaded on a 5% non-denaturing polyacrylamide gel made in 0.25% TBE. Electrophoresis was performed at 150 volts for 2 h and the gel dried onto a paper support and autoradiographed with an X-ray film. For the competition experiments, 1000-fold molar excess of unlabeled competitor oligonucleotide was added to the reaction mixture along with the labeled oligonucleotide.

DNA affinity precipitation assay (DAPA)

The 31-wt-S oligonucleotide biotinylated at the 5′ end (Operon Biotech., Inc.) was annealed with the anti-sense oligonucleotide. DAPA was performed as described previously (Billon *et al*., 1999) with some modifications. Briefly, the assays were done in a final volume of 400 μl of buffer D (20 mM HEPES, 10% glycerol, 50 mM KCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 10 μM $ZnCl_2$, 1 mM DTT and 0.25% Triton X100; pH 7.9), by mixing 4 μg of biotinylated double stranded 31-wt oligonucleotides with 20 – 30 μg of nuclear extracts. The mix was incubated on ice for 45 min and then was added to the buffer D with equilibrated Streptavidin coated Magnetosphere particles (SMPs) (Promega). The mixture was incubated at room temperature for 2 h with continuous agitation. SMPs were then captured using the magnetic stand and the supernatant removed without disturbing the SMPs pellet. Particles were washed four times with the buffer D and the final pellet obtained was resuspended in 2 × SDS – PAGE loading buffer and boiled for 5 min to uncouple the oligonucleoide bound proteins. After capturing the SMPs using the magnetic stand, the supernatant was loaded on SDS – PAGE gel and Western analysis was performed.

Immunoprecipitation and Western analysis

Immunoprecipitation of the whole cell extracts solubilized in RIPA buffer (1 × PBS, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail; pH 7.4) was performed as follows. Four hundred μg of whole cell extract was incubated with 10–15 μl of either anti-SP-1, anti-NF-1 or anti-Id-1 antibody (Santa Cruz Biotech.) in RIPA buffer on ice for 2 h and then 30 μl of protein-G-Sepharose (Sigma) was added to the reaction mixture and further incubation was given for 2 h at 4°C on a shaker. At the end of the incubation period, the sepharose beads were centrifuged and washed four times with TSA buffer (10 mM Tris-HCl, 150 mM NaCl, 0.25% sodium azide, and 0.1% Triton X100; pH 7.5), and then resuspended in 2 × SDS–PAGE buffer. Samples were then boiled for 5 min and loaded on SDS–PAGE gel and Western analysis was performed using anti-HDAC-1 (Santa Cruz Biotech.) and anti-Rb (Pharmingen) antibodies.

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